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Note

High-performance liquid chromatography of serum albumins on an N-methylpyridinium polymer-based column

KOICHI NISHIMURA, KUMIKO HARADA, SEIICHI MASUDA and ATSUSHI SUGII*

Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862 (Japan)

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Serum albumins, e.g. human serum albumin (HSA) and bovine serum albumin (BSA), are heterogenous with respect to sulphydryl content based on the conversion of mercaptalbumin into non-mercaptalbumin [1–3]. Mercaptalbumin in serum is equilibrated with its mixed disulphide with cysteine or glutathione (non-mercaptalbumin). The equilibrium is of a certain interest in connection with physiology and diagnosis, since the mercaptalbumin content changes in the elderly [4] as well as in some diseases [5].

Previously, we have reported on the application of an N-methylpyridinium polymer-based (4VP-EG-ME) column for high-performance liquid chromatography (HPLC) of proteins [6]. In the course of this study, it was found that the column showed good resolution of mercaptalbumin and non-mercaptalbumin. The resolution of both components of albumin by HPLC was first reported by Sogami and co-workers, who used Asahipack 520/520H [2–5] and 502N [4] columns, but these columns were useful only for HSA.

In this paper, the resolution behaviour of serum albumins from different sources (human, bovine, rabbit and rat) was investigated by using a 4VP-EG-ME column in ion-exchange HPLC.

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EXPERIMENTAL

Materials

Crystallized and lyophilized BSA (product No. A4378), essentially fatty acid-free BSA (No. A0281), fraction V HSA (No. A1653), essentially fatty acid-free HSA (No. A1887), rabbit serum albumin (RSA, No. A0639), rat serum albumin (RtSA, No. A6272), dithiothreitol (DTT) and oxidized glutathione were obtained from Sigma (St. Louis, MO, U.S.A.). Cystine was purchased from Kishida (Osaka, Japan).

Reduction of non-mercaptalbumin in BSA and HSA was carried out by adding DTT (3 mol per mol of BSA or HSA) in 0.1 *M* sodium phosphate buffer (pH 6.86) at 20°C [2]. Thus reduced albumin was directly applied to HPLC analysis without removing DTT.

Non-mercaptalbumin(Cys) and non-mercaptalbumin(Glu) were prepared by treatment of albumin with cystine [2] and oxidized glutatione [3], respectively. The non-mercaptalbumins prepared were applied to HPLC analysis without further treatment.

Human serum taken from patients with chronic renal failure before and after haemodialysis was applied undiluted to the 4VP-EG-ME column (injection volume 0.5 μl).

Chromatography

The HPLC system comprised a 655A-11 pump (Hitachi, Tokyo, Japan) equipped with a Hitachi L-5000 low-pressure gradient programmer and a Hitachi L-4000 variable-wavelength UV monitor. The column packing material, an N-methylpyridinium polymer cross-linked with ethylene glycol dimethacrylate, was prepared as described previously [6]. A 250 mm × 4 mm I.D. column, packed with the polymer beads (10–15 μm), was used. Unless stated otherwise, albumin was eluted with a 30-min linear gradient from 0 to 0.5 *M* sodium chloride in 0.05 *M* Tris–HCl buffer (pH 7) at a flow-rate of 0.5 ml/min. Chromatography was performed at room temperature with detection at 280 nm. No significant deterioration of the column was observed even after the continual use for three months under these conditions.

RESULTS AND DISCUSSION

Characterization of the peaks originating from HSA and BSA

HPLC analysis of serum albumin on the 4VP-EG-ME column was performed using a salt gradient elution. The four main peaks observed in the chromatograms of HSA and BSA (Fig. 1) were characterized according to the method of Sogami and co-workers [2,3]. DTT reduction of albumins resulted in disappearance of the second peak and an increase in the height of the first peak. Treatment of the albumin with cystine led to disappearance of the first

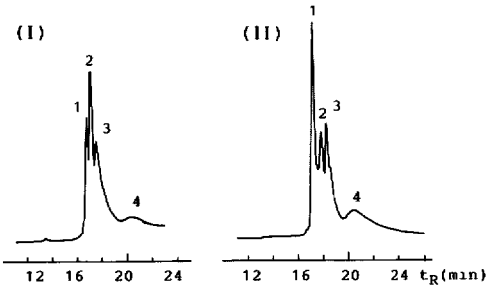

Fig. 1. Chromatograms of HSA (I) and BSA (II). Peaks: 1 = mercaptalbumin; 2 = non-mercaptalbumin; 3 = oxidized form; 4 = polymerized albumin. Column, 4VP-EG-ME (250 mm × 4 mm I.D.); elution, 30-min linear gradient of sodium chloride from 0 to 0.5 *M* in 0.05 *M* Tris–HCl (pH 7) at a flow-rate of 0.5 ml/min.

peak and an increase in the height of second peak. These facts indicated that the first peak was mercaptalbumin and the second peak non-mercaptalbumin coupled with cysteine. Since the third peak was unaltered by DTT reduction or by treatment with cystine or oxidized glutathione, this peak is likely to be an oxidized form of mercaptalbumin containing a sulphinic acid/sulphonic acid group, as reported in refs. 4, 7 and 8. The fourth peak was assigned to an oligomeric albumin fraction, including the dimeric form, by comparison with the chromatogram of an authentic sample prepared by the oxidation of albumin with the use of cupric chloride [2].

A comparison of defatted and non-defatted albumin indicated that the lipid impurities strongly bound to albumin scarcely influenced the HPLC elution profile, except for a small change in the oligomeric albumin peak.

Effect of buffer system on peak resolution

The resolution of mercaptalbumin and non-mercaptalbumin in HSA and BSA is strongly affected by the pH of the mobile phase (Fig. 2). The best resolution was obtained at pH 6.5–7.0. It is assumed that below pH 5 albumin has insufficient negative charge for retention on the column. The retention time of albumin increases with increasing pH of the mobile phase, and the resolution between mercaptalbumin and non-mercaptalbumin was poor at higher pH. The retention behaviour of these albumins is probably influenced by conformation changes of the protein caused by the alteration of eluent pH. In the most suitable pH region, the resolution of BSA was better than that of HSA.

The effect of a buffer system as eluent on the resolution of mercaptalbumin and non-mercaptalbumin was examined by using various sources of albumin (human, bovine, rabbit and rat). Fig. 3 shows the chromatograms of these albumins. Human, bovine and rabbit serum albumins could be resolved into their components in all the buffer systems used. In the case of RtSA, however,

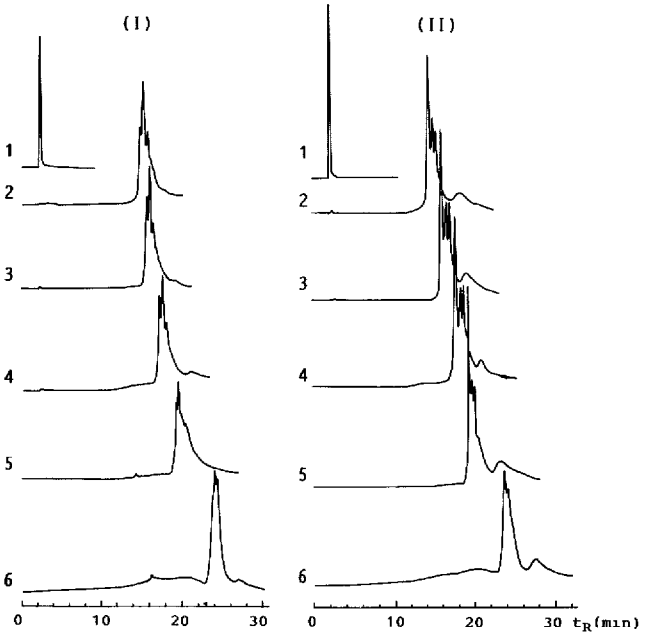

Fig. 2. Chromatograms of HSA (I) and BSA (II) at various eluent pH values: 1 = pH 5.0; 2 = pH 6.0; 3 = pH 6.5; 4 = pH 7.0; 5 = pH 7.5; 6 = pH 8.12. Other conditions as in Fig. 1.

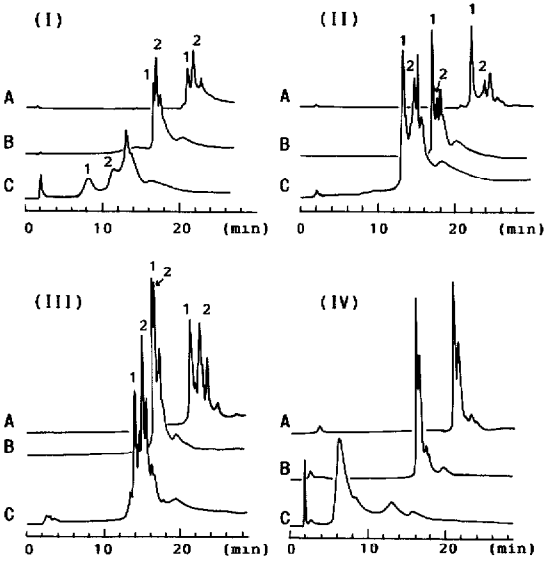

Fig. 3. Chromatograms of HSA (I), BSA (II), RSA (III) and RtSA (IV) with different eluent buffers: (A) 0.05 *M* Tris-acetic acid (pH 7) (gradient, CH_3COONa 0 to 0.5 *M*); (B) 0.05 *M* Tris-HCl (pH 7) (gradient, NaCl 0 to 0.5 *M*); (C) phosphate buffer (pH 7) 0.05 *M* to 0.5 *M*. Gradient time, 30 min linear. Peaks: 1 = mercaptalbumin; 2 = non-mercaptalbumin (Cys). Other chromatographic conditions as in Fig. 1.

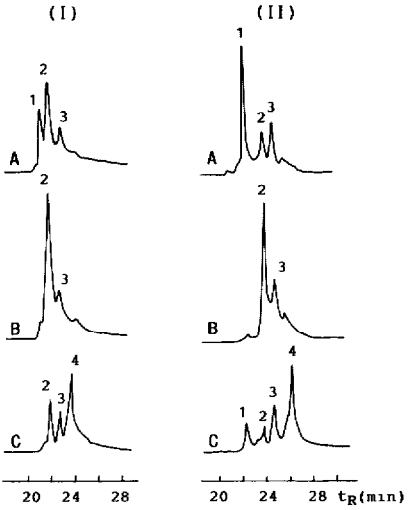

Fig. 4. Chromatograms of non-mercaptalbumin (Cys) and non-mercaptalbumin (Glu) of HSA (I) and BSA (II). (A) Non-treated albumin; (B) albumin treated with cystine; (C) albumin treated with oxidized glutathione. Peaks: 1 = mercaptalbumin; 2 = non-mercaptalbumin (Cys); 3 = oxidized form; 4 = non-mercaptalbumin (Glu). Albumins were eluted using a 30-min linear gradient of sodium acetate from 0 to 0.5 *M* in 0.05 *M* Tris-acetic acid (pH 7) at a flow-rate of 0.5 ml/min. Other chromatographic conditions as in Fig. 1.

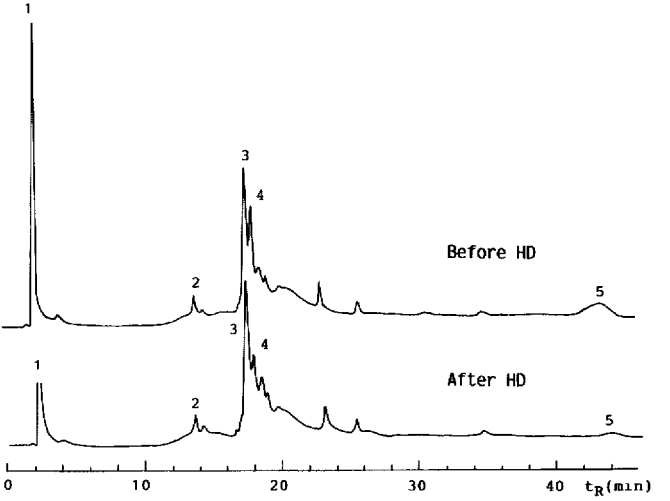

Fig. 5. Typical chromatograms of haemodialysis patient serum before and after haemodialysis (HD). Peaks: 1 = γ-globulin; 2 = transferrin; 3 = mercaptalbumin; 4 = non-mercaptalbumin (Cys); 5 = uric acid. Linear gradient elution with increasing sodium chloride from 0 to 0.5 *M* (0-30 min from 0 to 0.5 *M*, 30-60 min isocratic elution of 0.5 *M*) in 0.05 *M* Tris-HCl (pH 7).

clear resolution of the components was not observed in all buffer systems. The chromatograms of RtSA obtained using Tris–HCl and Tris–acetic acid buffer systems showed two main peaks. However, the first peak was not reduced by the cystine treatment, and the height of the second peak decreased only slightly with DTT reduction. Therefore, it is assumed that the first peak may contain both mercaptalbumin and non-mercaptalbumin. In the case of phosphate buffer, the peaks of HSA and RtSA became broad compared with those of BSA and RSA, and all albumins tested showed sharp peaks in buffer systems other than phosphate. Reproducible chromatograms were obtained in every buffer system. The best separation of the mercaptalbumin and non-mercaptalbumin components of HSA, BSA and RSA was achieved in Tris–acetic acid buffer. In this system, however, the oligomer fraction, present as the final peak in other buffer systems, was not detected.

Separation of non-mercaptalbumin(Cys) and non-mercaptalbumin(Glu)

It was reported by King [9] and Andersson [10] that human non-mercaptalbumin (HNA) and bovine non-mercaptalbumin (BNA) are composed of two kinds of mixed disulphide, i.e. mixed disulphides with cysteine and glutathione. Our column showed satisfactory resolution of the BNA(Cys) and BNA(Glu) peaks or the HNA(Cys) and HNA(Glu) peaks (Fig. 4). The chromatograms indicate that commercial albumin preparations contain only very small amounts of non-mercaptalbumin(Glu). The chromatograms further show that a part of the non-mercaptalbumin(Cys) was converted into non-mercaptalbumin(Glu) by the treatment with excess oxidized glutathione. The Asahipak GS-520H [3] and ES-502N [4] columns gave incomplete separation of the two non-mercaptalbumins in HSA. The good resolution of the mixed disulphides obtained with the 4VP-EG-ME column shows how retention may be influenced by small changes in the albumin structure.

Chromatograms of human serum

Our column was further applied to the analysis of human sera from haemodialysis patients with chronic renal failure. As shown in Fig. 5, components of albumin in the serum eluted at ca. 17.5 min and gave two peaks consisting of mercaptalbumin and non-mercaptalbumin(Cys). These peaks did not interfere with other components in the serum. γ-Globulin and transferrin eluted at 2.0 and 13.8 min, respectively. The components of albumin in serum could be analysed by direct injection of serum on the column. Sogami and co-workers [3,5] reported that the amount of mercaptalbumin in serum of haemodialysis patients increased after haemodialysis. The same tendency was observed in this study.

In conclusion, the 4VP-EG-ME column showed good resolution of albumin components [mercaptalbumin, non-mercaptalbumin(Cys), non-mercaptal-

bumin (Glu) and oligomeric albumins]. This suggests that it can be applied to studies on the interaction between albumin and pharmaceuticals reactive to the SH group. Application to the diagnosis of some diseases associated with a change of albumin components also seems promising.

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